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A novel purification of ferric citrate receptor (FecA) from *Escherichia coli* UT5600 and further characterization of its binding activity

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In our earlier paper, it was demonstrated that the FecA receptor protein from *Escherichia coli* UT5600/pBB2 (*leu*$^-$, *proC*$^-$, *trpE*$^-$, *entA*$^-$, *rpsl*$^-$, Δ(*ompT-fepA*) /*Ampr*, *fepA*) binds with ferric enterobactin. In order to explore this further the outer membrane receptor protein, FecA, has been isolated from UT5600 (*fepA*$^-$) and purified to homogeneity by DE-52–cellulose anion exchange chromatography followed by MonoPFPLC chromatofocusing. Partially purified FecA and homogeneous FecA show binding activity to [55Fe]ferric enterobactin and the binding is specific. Binding activity of FecA can be enhanced by ferric citrate. Lipopolysaccharide-free FecA as ascertained by silver staining and the endotoxin test still retains the same activity. *In vivo* uptake studies using different strains of *E. coli* suggest that FecA in *E. coli* plays an important role in ferrienterobactin transport.

Keywords: binding activity, ferric citrate binding protein, FPLC chromatofocusing, iron uptake, purification of membrane protein

Introduction

The inducible citrate-dependent iron transport system in *Escherichia coli* was found in 1973 by using different mutant strains (Frost & Rosenberg 1973). This iron transport system is repressed by Fur and induced by iron–citrate in the medium (Hussein *et al.* 1981, Wagegg & Braun 1981, Zimmermann *et al.* 1984). Citrate does not serve as a carbon or energy source in *E. coli* and it is not transported under aerobic growth conditions (Hussein *et al.* 1981). However, citrate and iron have to enter only the periplasmic space in order to induce this citrate-dependent iron transport system (Van Hove *et al.* 1990). It was demonstrated that the transport system includes an outer membrane receptor FecA with a 'TonB box' in the N-terminal region, a precursor protein FecB found in the periplasm and a membrane-bound protein FecE (Pressler *et al.* 1988). A recent study has indicated that two very hydrophilic proteins, FecC and FecD in the cytoplasmic membrane, are also involved in this uptake system (Staudenmaier *et al.* 1989). Two membrane-associated regulatory proteins, FecI and FecR, are required for induction (Van Hove *et al.* 1990). More recently, an investigation indicated that FecE is a membrane associated ATP-binding protein (Schultz-Hauser *et al.* 1992). These observations, therefore, suggest that the citrate-mediated iron uptake through the outer membrane is mediated by TonB and energized by the proton motive force of the cytoplasmic membrane, and that the uptake through the cytoplasmic membrane is energized by ATP hydrolysis (Braun *et al.* 1992).

FecA protein is thus one of the outer membrane receptor proteins in the iron uptake system. Therefore it is important to investigate the function and structure of the protein. In our earlier paper (Zhou *et al.* 1993), the first purification of FecA from UT5600/pBB2 was described and the binding behavior of this protein was found to be similar to FepA, the ferric enterobactin receptor in *E. coli*. In order to explore this further, a novel purification procedure of FecA is described while the protein is obtained from UT5600 (FepA$^-$) to eliminate any possible contamination of FepA. The binding activity of FecA to ferric enterobactin and the enhancement of this binding activity by citrate is reported.

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Materials and methods

Materials

DE-52 (DEAE-cellulose) was obtained from Whatman Biosystems (Maidstone, Kent, UK). MonoP, Sephadex G-10, Sephadex G-25, Sephadex LH-20 and polybuffer 74 were purchased from Pharmacia LKB (Sollentura, Sweden). [^{55}Fe]Ferric chloride (39 mCi mg^{-1} in 0.5 M hydrochloride acid) was obtained from Dupont NEN (Boston, MA, USA).

Microorganisms and culture conditions

E. coli strains UT5600 (*leu*$^-$, *proC*$^-$, *trpE*$^-$, *rpsL*$^-$, *entA*$^-$, Δ(*ompT-fepA*)$^-$; UT5600/pBB2 (*leu*$^-$, *proC*$^-$, *trpE*$^-$, *rpsL*$^-$, *entA*$^-$, Δ(*ompT-fepA*$^-$/*Ampr, fepA*) were kindly provided by Dr M. D. Lundrigan (University of Mississippi, Medical Center, Jackson, MS). *E. coli* strains AN102 and AN311 were provided by Dr J. B. Neilands (University of California at Berkeley, CA) and Dr G. Winkelmann (University of Tübingen, Germany), respectively.

Strain UT5600 was grown in TY medium to an optical density at 660 nm of 0.300 at 37 °C. The cell suspension in TY medium was stored frozen at −70 °C in 1 ml quantities as a 30% solution in glycerol until ready to use. For FecA production, the cells thawed at room temperature were inoculated in 50 ml of TY medium and grown at 37 °C to an OD of 0.15 at 660 nm. The cell suspension was transferred to 1000 ml of minimal growth medium in 2 l Fernbach flasks and grown at 37 °C for 16–18 h. Minimal culture medium contained per liter of distilled water 10.5 g K_2HPO_4; 4.5 g KH_2PO_4; 1.0 g $(NH_4)_2SO_4$; 0.5 g sodium citrate (dehydrate); 0.25 g $MgSO_4 \cdot 7H_2O$; 0.01% L-proline; 0.01% L-leucine; 0.01% L-tryptophan; 0.2% glucose and 25 μg thiamine-HCl (vitamin B_1). After harvest by centrifugation, the cells were washed once with buffer A (50 mM Tris-HCl buffer containing 10 mM benzamidine with a pH of 7.5).

FecA purification

The purification of FecA from *E. coli* UT5600 was performed in three steps at room temperature.

Preparation of crude extract. The procedure for the preparation of FecA from outer membrane of extract was similar to that for FepA (Jalal & van der Helm 1989). Fractions rich in FecA examined by SDS-PAGE were pooled and dialyzed against 1 l of buffer B (25 mM Bis-Tris-HCl, 10 mM benzamidine, 1% Triton X-100, pH 7.5).

DEAE-cellulose DE-52 column chromatography. Dialyzed solution (60 ml) was applied to a FPLC-DE-52 column (1 × 26 cm) previously equilibrated with buffer B. FecA protein was eluted at flow rate of 0.2 ml min^{-1} with a 160 ml FPLC gradient from 0 to 10% and 20% of 1 M NaCl in buffer B. The purification profile of FecA was monitored at 280 nm. FecA protein was determined by a binding assay and SDS-PAGE according to known mobility of FecA (Zhou *et al.* 1993). FecA active fractions were pooled and exhaustively dialyzed against buffer C (25 nm Bis-Tris, 5 mM Benzamidine, 1% Triton X-100, pH 7.1 adjusted by saturated iminodiacetic acid).

FPLC chromatofocusing. Partially purified FecA receptor (12 ml) was applied to a Pharmacia MonoP column (5/20) pre-equilibrated with buffer C. The proteins were eluted with buffer D (10% of polybuffer 74; 1% Triton X-100, 5 mM benzamidine, pH 4.0 adjusted by saturated iminodiacetic acid). FecA active fractions were determined by binding assay and SDS-PAGE. The polybuffer was removed by centriprep. Complete removal of polybuffer was determined by checking the pH value of the solution.

SDS-PAGE. SDS-PAGE was carried out on 10 and 7% polyacrylamide slab gels, respectively, by modification of the Laemmli buffer system (Laemmli 1970). Coomassie brilliant blue R-250 was employed for staining.

N-terminal sequence determination of receptor

This was carried out by the method of Matsudaira (1987). After SDS slab-gel electrophoresis of a 30 μg purified protein, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine) with 0.1% SDS for 15 min. During this period of time a PVDF membrane was rinsed with 100% methanol for 5 s and stored in transfer buffer. The gel, sandwiched between a sheet of PVDF membrane and several sheets of blotting paper, was assembled into a blotting apparatus (BioRad) and electransferred overnight at 80 V (0.3 A). The PVDF membrane was washed in deionized water for 5 min, stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol for 5 min and then destained in 50% methanol, 10% acetic acid for 10 min at room temperature. The membrane was finally rinsed in distilled water for 10 min to remove glycine and Tris, and air dried. The protein band was cut out with a clean razor and either loaded onto the sequenator or stored at −20 °C.

The membrane with the protein band was centered on the Teflon seal and placed in the cartridge of the sequenator. Protein was sequenced on an Applied Biosystems Sequenator (Model 470) equipped with online phenylthiohydantion (PTH) analysis using the regular program O3RPTH. The PTH-amino acid produced at each cycle was converted automatically and identified in a model 120-A amino acid PTH analyzer. Each HPLC chromatogram of amino acid PTH output by the instrument was analyzed according to the retention times of standard PTH-amino acid derivatives.

Protein determination

Protein content was measured by a modification of the Lowry procedure (Lowry *et al.* 1952; Dudley & Grieve 1975). Bovine serum albumin was used as the standard.

Purification of enterobactin

Enterobactin was purified using the procedure described before (Zhou *et al.* 1993). Briefly, crude enterobactin extracted from the supernatant of *E. coli* AN 311 culture was partially purified with Sephadex LH-20 and further purified with a FPLC reverse phase column (PepRPC 10/20). The purity of enterobactin was determined by silica gel thin-layer chromatography.

Preparation of ferric–ligand complexes

[^{55}Fe]Ferric enterobactin and non-labelled ferric enterobactin were prepared and purified as reported earlier (Zhou *et al.* 1993). Ferric citrate (100 mM) was prepared by mixing equi-amounts of 400 mM $FeCl_3$ with 200 mM sodium citrate at pH 6.0, because the (uncertain) stability of ferric citrate is low (10^{25} at pH 7) (Sillen & Martell 1964), a 2-fold concentration of $FeCl_3$ was employed to saturate citrate.

Binding assay of FecA

This was done using the same procedure as for the assay of FepA binding activity (Zhou *et al.* 1993). First, 10 μg of partially purified or purified FecA from UT5600 and 0.3 μM of [^{55}Fe]ferric enterobactin were introduced to a reaction buffer (0.1 M Tris–HCl buffer with a pH of 7.4 containing 0.1% Triton X-100) in a total volume of 200 μl. Then, for inhibition studies, 20 μM of non-labeled ferric enterobactin was added to the reaction solution. An additional experiment was performed in order to investigate the effects of citrate on the binding activity of FecA with ferric enterobactin; 20 μM of ferric citrate was added to the reaction solution and the binding activity of the receptor was determined by the area of the radioactive peak.

Silver staining

For detecting lipopolysaccharide (LPS) in SDS–PAGE, silver staining was performed by the methods of Tsai & Frasch (1982) and Nielsen & Brown (1984).

Quantification of LPS

LPS in the samples was measured using the E-Toxate test (Sigma, St Louis, MO). Endotoxin (LPS from the walls of Gram-negative bacteria) and LPS from *E. coli* D31m4 (List Biological,) were used as the references.

Iron transport studies

This was carried out as described by Pugsley and Reeves (1976).

Preparation of active cells. Bacteria were grown overnight in minimal medium, washed twice in iron-free medium by centrifugation and subcultured in iron-free medium to an OD_{660} of 0.01. Subcultured cells were grown to an OD_{660} of approximately 0.2 with or without citrate, harvested by centrifugation (3500 × *g* for 20 min), washed twice in iron-free medium and finally resuspended in iron-free medium to an OD_{660} of 0.8–1.0. Cells were chilled to 4 °C.

Iron uptake. Assays were performed at 37 °C in sterile polystyrene tubes with vigorous shaking. At zero time, 1.5 ml of minimal medium containing [^{55}Fe]enterobactin (2 μM) was mixed with 1.5 ml of cells (1.5–2 × 10^9) in uptake medium. Each sample (0.5 ml) taken from the mixed suspension at intervals was diluted into 3 ml of 10 mM EDTA (pH 7.0) and then filtered immediately through membrane filters (Millipore, pore size 0.45 μm), which had been presoaked in 10 mM EDTA. The filters were washed twice with 10 ml of 0.85 (w/v) NaCl. The thoroughly dried filters were weighed, placed in scintillation vials and counted after addition of scintillation fluid.

Results

Purification of FecA

Table 1 summarizes the purification of FecA from *E. coli* UT5600. The FecA receptor can be purified from the outer membrane extract of *E. coli* to an homogeneous form using two steps of chromatography. The *fepA*$^-$ mutant, UT5600, produces FecA, the production of which can be enhanced by adding citrate. The FecA yield after DE-52 chromatography is about 13% of total protein. The second step is a chromatofocusing column where proteins are separated on the basis of their isoelectric point. It is a method which generally achieves good resolution and also, in the case of proteins solubilized in detergents, seems to be more effective than other separation techniques (Garavito & Rosenbusch 1986, Latsch *et al.* 1992). Figure 1 shows the profile for the FecA purification on a MonoP FPLC chromatofocusing column. The yield for FecA on this column is rather low. The reason for that is the reduced solubility of the membrane protein at the pH close to the p*I* point and 50–75% of the protein is therefore lost. The p*I* point of FecA is 5.15. The overall yield of FecA is about 2%.

Table 1. Purification of FecA from *E. coli* UT5600

Step	Vol (ml)	Protein content (mg ml^{-1})	Total protein (mg)	Yield (%)
Extract	64	3.2	204	100
DE-52	12	2.3	27	13
Mono-P	1	3.5	4	2

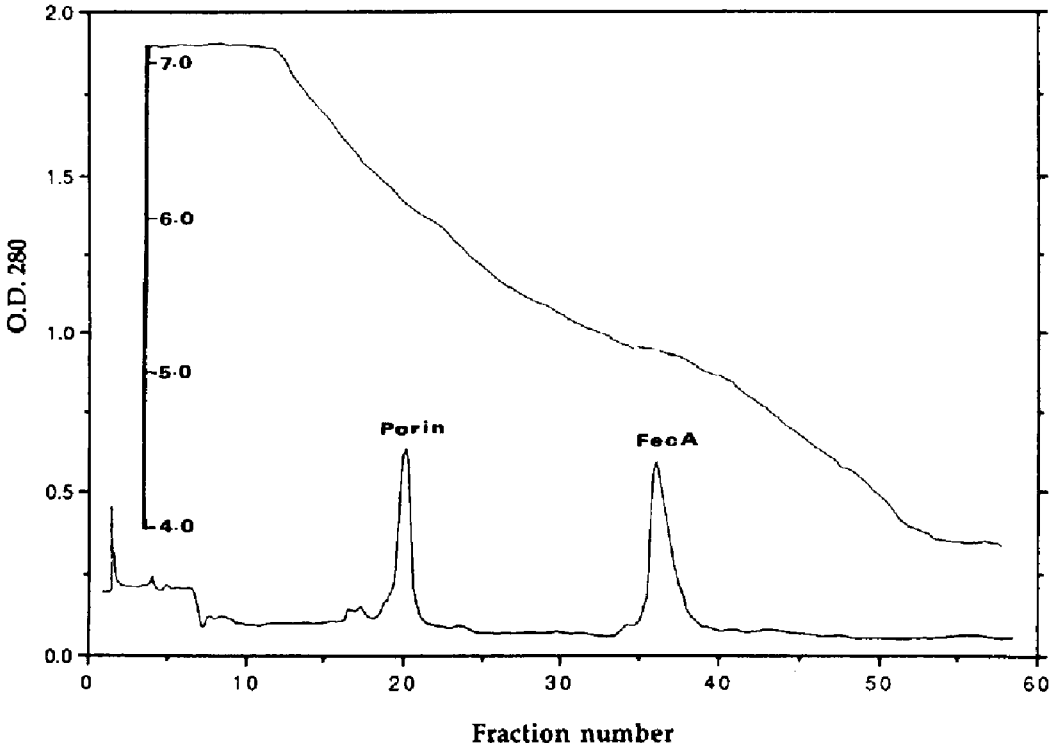

Figure 1. Chromatofocusing of FecA from *E. coli* UT5600, purified by DE-52 ion exchange chromatography. FecA was eluted at pH 5.15 from the MonoP column.

Purity of FecA

The purity of FecA was examined by 10 or 7% SDS–PAGE according to the known mobility of FecA (Zhou *et al.* 1993). After staining with Coomassie blue, 1 and 2 μg of purified FecA (lanes C1 and C2 in Figure 2, respectively) show a single band, indicating the purity of FecA. *E. coli* UT5600 is a *fepA*$^-$ mutant and should not produce FepA. The identity of the protein as FecA is proven by determining the N-terminal sequence. This sequence is shown to be AQVNIAP, which corresponds to the sequence determined for FecA (Pressler *et al.* 1988).

Receptor binding activity

The binding activities of partially purified FecA receptor, after ion exchange chromatography, and purified FecA, after chromatofocusing, are shown in Figure 3. The methodology has been described (Zhou *et al.* 1993). The elution profile from the Sephadex G-25 column when loaded with [^{55}Fe]ferric-enterobactin by itself is shown in panel A. The elution profiles when [^{55}Fe]enterobactin is mixed with partially purified FecA and purified FecA are shown in panels B and E, respectively. The ratio on a molar scale of [^{55}Fe]ferric enterobactin to FecA is 1:2. It shows that both preparations of FecA bind

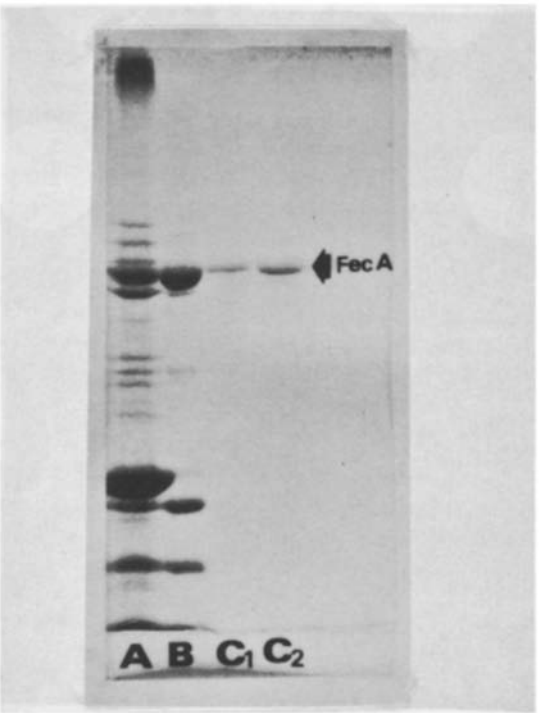

Figure 2. SDS–PAGE of purified FecA from *E. coli* UT5600. Samples were solubilized in sample buffer at 100 °C for 5 min. Gels were stained by the Coomassie brillant blue R-250 staining method. Full details are given in the Discussion section.

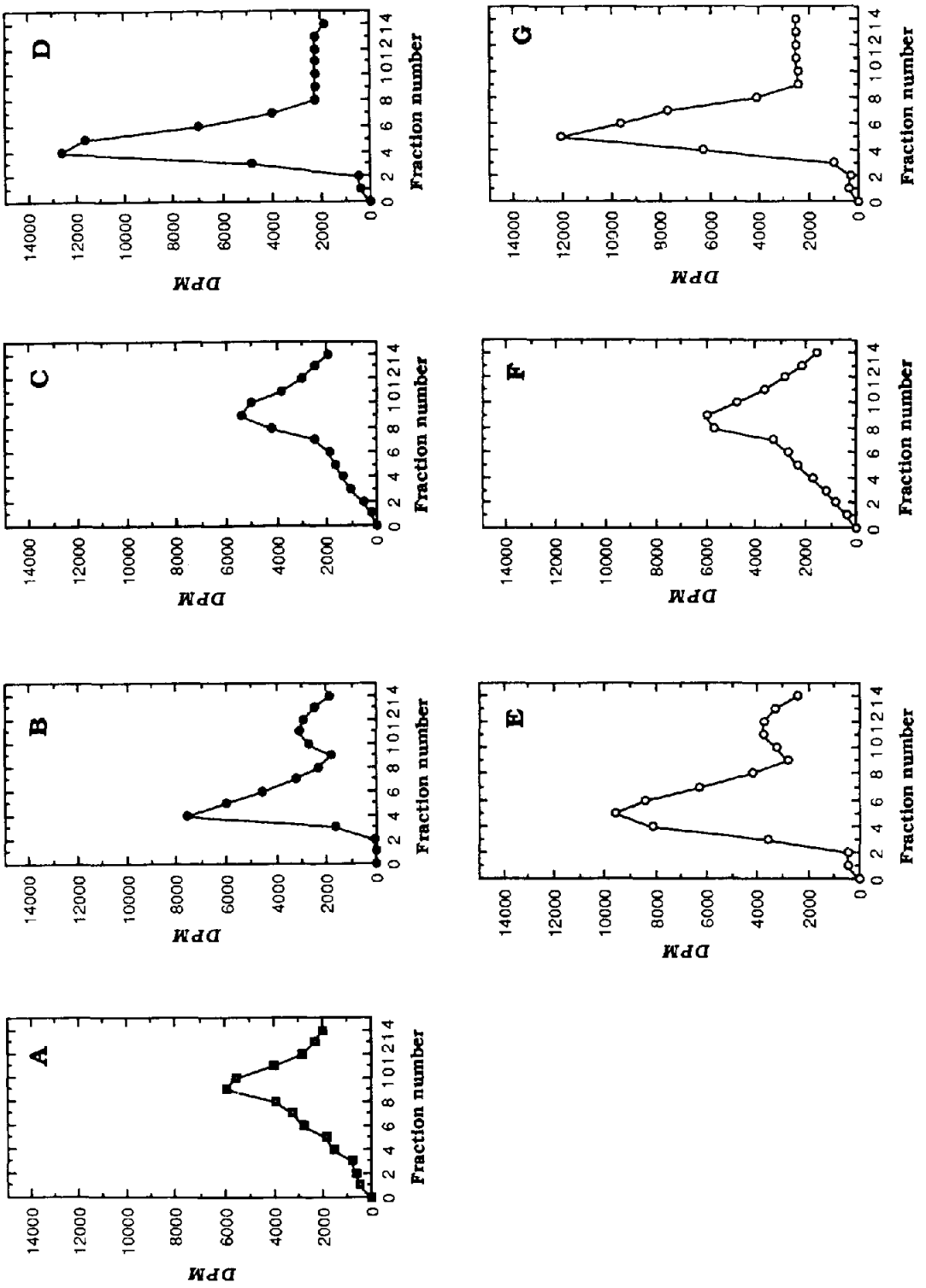

Figure 3. Determination of binding activity of partially purified and purified FecA from outer membrane extraction of *E. coli*: (**A**) [^{55}Fe]enterobactin alone; (**B**) [^{55}Fe]enterobactin plus partially purified FecA after DE-52 column; (**C**) [^{55}Fe]enterobactin plus partially purified FecA mixed with cold ferric enterobactin; (**D**) [^{55}Fe]enterobactin plus partially purified FecA mixed with ferric citrate; (**E**) [^{55}Fe]enterobactin plus purified FecA after Mono P column; (**F**) [^{55}Fe]enterobactin plus purified FecA mixed with cold ferric enterobactin; (**G**) [^{55}Fe]enterobactin plus purified FecA mixed with ferric citrate.

[^{55}Fe]enterobactin. Panels C and F show the elution profiles when labeled [^{55}Fe]enterobactin is mixed with unlabeled enterobactin (ratio 1:66) and both preparations of FecA. It shows that the binding is specific. Panels D and G show the elution profile when ferric citrate is added to the binding mixtures, consisting of [^{55}Fe]enterobactin and both preparations of FecA. It shows that in both cases citrate enhances the binding between receptor protein and the siderophore.

LPS has been determined in both preparations of FecA. This was done by the silver staining method on SDS–PAGE (Tsai & Frasch 1982, Nielsen & Brown 1984) and the E-Toxate test. The first method (results not shown) indicates that crude FecA (after solubilization) and partially purified FecA (after ion exchange chromatography) contain a measurable amount of LPS, while LPS is completely removed from FecA after chromatofocusing. The E-Toxate test shows that 0.1 ml of purified FecA containing 100 μg protein reacts with the E-Toxate working solution to form a hard gel, indicating either the absence of LPS or an amount less than 0.015 EU ml^{-1} in the FecA sample, while extracted FecA (100 μg protein) or partially purified FecA (100 μg protein) do not form even a soft gel, showing the LPS content to be above 0.5 EU ml^{-1}. The results of the binding assays for partially purified and purified FecA therefore show that the presence of LPS has a minor but not a profound effect on the binding between the membrane protein and the siderophore.

Uptake studies

Figure 4 shows the time-dependent kinetic uptake of [^{55}Fe]ferric enterobactin in both strains, UT5600/pBB2 and UT5600. UT5600/pBB2 produces both FepA (plasmid) and FecA (chromosome) while UT5600 produces FecA but no FepA. It should be noted that UT5600 assimilates [^{55}Fe]enterobactin despite the fact that it does not produce FepA. This effect, however, has been seen before (McIntosh *et al.* 1978, 1979), but has been left unexplained. The UT5600/pBB2 strain shows a greater uptake rate for [^{55}Fe]enterobactin. When citrate is used to induce a greater production of FecA in both strains, one observes an approximate doubling of the uptake for both strains.

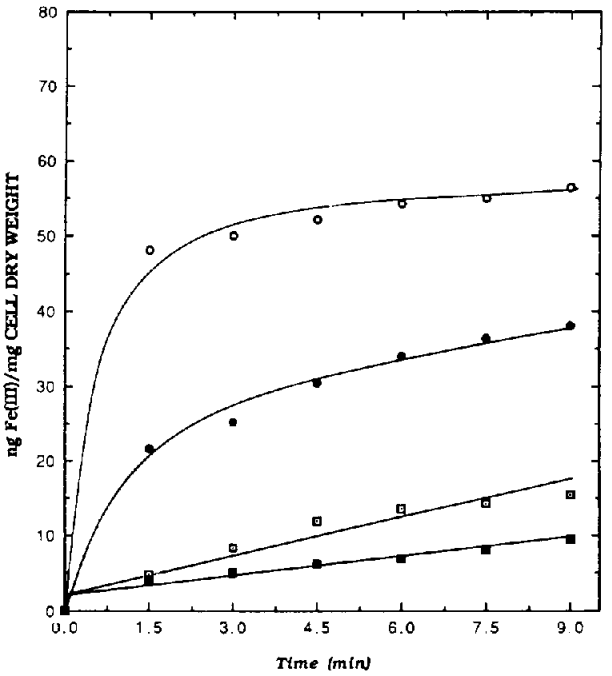

Figure 4. Ferric enterobactin uptake by strains UT5600 (squares) and UT5600/pBB2 (circles) in MM media. Open and closed symbols indicate the presence and absence of citrate in the uptake medium, respectively.

Discussion

FepA is the outer membrane receptor protein for ferric enterobactin. This has been proven with transport studies and binding experiments (Hollifield & Neilands 1978, Neilands *et al.* 1980; Fiss *et al.* 1982, Ecker *et al.* 1986, Ozenberger *et al.* 1987). The FecA outer membrane receptor protein is used for uptake by ferric citrate (Wagegg & Braun 1981). In our earlier paper, we show, however, that both FecA and FepA, separately purified from UT5600/pBB2, bind ferric enterobactin and their binding is specific. UT5600/pBB2 produces both proteins when induced by citrate. In order to eliminate the possibility of contamination by FepA in the FecA preparations, the *fepA*$^-$ mutant UT5600 is used in the present communication to produce FecA. The purification of FecA is shown to be reproducible but the yield is relatively small.

Membrane proteins in *E. coli* are located in bilayers of lipids such as phospholipid, LPS and other lipids. However, the major structural component in the outer membrane of Gram-negative bacteria is LPS (Gennis 1989). LPS plays an important role as a permeation barrier, making the bacteria resistant to a number of antibiotics and supporting the conformational structures of membrane proteins (Gennis 1989). A recent investigation indicates that purified porin from *Salmonella minnesota* loses its conductivity and activity to form channels after the LPS has been removed by

chromatofocusing (Latsch *et al.* 1992). On the other hand, the structures of three porins have recently been determined (Weiss *et al.* 1990, Pauptit *et al.* 1991, Cowan *et al.* 1992). All these proteins were purified using chromatofocusing for removal of LPS (Garavito & Rosenbusch 1986, Pauptit *et al.* 1991). The structural conformation and aggregation for the porins as determined by single crystal X-ray diffraction are considered to be closely similar to the structures in the intact membrane *in vivo*. In our present studies, the binding assays show that the LPS does not affect the binding behavior of FecA significantly. FecA, therefore, does not require LPS for its structural integrity and consequently a possible structure determination by single crystal X-ray diffraction would be meaningful.

In the previous communication we have reported that not only FepA but also FecA binds ferric enterobactin and that for both the binding is specific. Our present experiments confirm our earlier results for FecA and show that purified FecA from UT5600 has binding activity for [^{55}Fe]ferric enterobactin and that the binding is specific. The additional result we report here is that the binding activity of FecA is increased by adding ferric citrate to the reaction solution. A possible explanation for the increase of binding activity of FecA by citrate is that the citrate changes the conformation of FecA, thereby improving its binding capacity. FecA may have a specific site for citrate and binding of citrate to FecA may lead to a conformational change of FecA, increasing the binding activity for ferric enterobactin.

The mechanism of binding *in vitro* may not be identical to the process involved in uptake kinetics *in vivo*. In order to explore the difference, uptake studies are required. UT5600/pBB2 is a plasmid-encoded *fepA* strain while UT5600 is a *fepA*$^-$ mutant. Both of them are *fecA* normal and *entA* deletion-type organisms. Thus, both UT5600 and UT5600/pBB2 can only utilize environmental enterobactin as an iron chelator for iron transport. The results for UT5600/pBB2 show a significant uptake of ferric enterobactin as can be expected. However, UT5600 also shows uptake of ferric enterobactin although at a considerable lower rate. This indicates that binding is indeed different from uptake because both FecA and FepA show similar binding behavior. On the other hand, it shows just as well that FecA is able to transport ferric enterobactin but at a low rate. The indication for involvement of FecA in ferric enterobactin transport can also be found in the literature. Earhart's group (McIntosh *et al.* 1978) reported that RW193 (*ent*$^-$) takes up iron from ferric enterobactin rapidly; however, UT2300 (*fepA*$^-$, *ent*$^-$) derived from RW193 still takes up ferric enterobactin. This may suggest that FepA is not the only receptor responsible for uptake of ferric enterobactin. When citrate is used to induce FecA production, the uptake rates of both UT5600 and UT5600/pBB2 approximately double. It is unlikely that [^{55}Fe]ferric citrate is transported because all iron is bound to the much more stable ferric enterobactin (stability constant for ferric enterobactin = 10^{52} and for ferric citrate = 10^{25}) (Harris *et al.* 1981, Sillen & Martell 1964). The increase in uptake by UT5600 indicates, once again, that FecA is involved in ferric enterobactin transport. It also can explain the observation made by Raymond's group (Ecker *et al.* 1986) that, when RW193, an enterobactin synthesis deficient mutant (*pro*$^-$, *leu*$^-$, *trp*$^-$, *purE*$^-$, *entA*$^-$), was grown in an iron-deficient medium supplemented with 5 mM ferric enterobactin, no growth was observed unless citrate was added to the medium. The fact that doubling of uptake occurs for both UT5600 and UT5600/pBB2 means that a larger increase in uptake rate occurs for UT5600/pBB2 than for UT5600. The only apparent explanation for this behavior is that a cooperative mechanism between FepA and FecA is operative when both are produced as in UT5600/pBB2.

We have shown that both FepA and FecA bind ferric enterobactin and that both transport the siderophore *in vivo*. We have also shown that citrate increases the binding of FecA with ferric enterobactin and that citrate increases the uptake rates in strains which either do or do not produce FepA. Obviously, further investigations are required to characterize the mechanism of transport using *fecA*$^-$ mutants. Both FepA and FecA retain their integrity when purified and a structure determination of either or both will be a meaningful enterprise.

Acknowledgments

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